PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/395, 38/17, 35/14, 35/28 // (A61K 39/395, 35:14) (A61K 39/395, 35:28) (A61K 38/17, 35:14)

A1

(11) International Publication Number: **WO 95/06481**

(43) International Publication Date: 9 March 1995 (09.03.95)

(21) International Application Number: PCT/US94/09953

(22) International Filing Date: 2 September 1994 (02.09.94)

(30) Priority Data:
08/116,255 2 September 1993 (02.09.93) US
08/232,929 25 April 1994 (25.04.94) US

(60) Parent Applications or Grants
(63) Related by Continuation
US 08/116,255 (CIP)
Filed on 2 September 1993 (02.09.93)
US 08/232,929 (CIP)
Filed on 25 April 1994 (25.04.94)

(71) Applicant *(for all designated States except US)*: TRUSTEES OF DARTMOUTH COLLEGE [US/US]; Hanover, NH 03755 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NOELLE, Randolph, J. [US/US]; Rural Route 3, Box 257, Cornish, NH 03745 (US). FOY, Teresa, M. [US/US]; 3-13 Meadowbrook Village, Lebanon, NH 03756 (US). DURIE, Fiona, H. [GB/US]; 1731 N.W. 58th #101, Seattle, WA 98107 (US).

(74) Agents: MANDRAGOURAS, Amy, E. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR INDUCING ANTIGEN-SPECIFIC T CELL TOLERANCE

(57) Abstract

Methods for inducing antigen-specific T cell tolerance are disclosed. The methods involve contacting a T cell with: 1) a cell which presents antigen to the T cell, wherein a ligand on the cell interacts with a receptor on the surface of the T cell which mediates contact-dependent helper effector function; and 2) an antagonist of the receptor on the surface of the T cell which inhibits interaction of the ligand on the antigen presenting cell with the receptor on the T cell. In a preferred embodiment, the cell which presents antigen to the T cell is a B cell and the receptor on the surface of the T cell which mediates contact-dependent helper effector function is gp39. Preferably, the antagonist is an anti-gp39 antibody or a soluble gp39 ligand (e.g., soluble CD40). The methods of the invention can be used to induce T cell tolerance to a soluble antigen or to an allogeneic cell. The methods of the invention can also be used to induce tolerance in cases of bone marrow transplantation and other organ transplants and to inhibit graft-versus-host disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

METHODS FOR INDUCING ANTIGEN-SPECIFIC T CELL TOLERANCE

Background of the Invention

To induce antigen-specific T cell activation and clonal expansion, two signals provided by antigen-presenting cells (APCs) must be delivered to the surface of resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165, 302-319; Mueller, D.L., et al. (1990) *J. Immunol.* 144, 3701-3709; Williams, I.R. and Unanue, E.R. (1990) *J. Immunol.* 145, 85-93). The first signal, which confers specificity to the immune response, is mediated via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Schwartz, R.H. (1990) *Science* 248, 1349-1356). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. (1988) *J. Immunol.* 140, 3324-3330; Linsley, P.S., et al. (1991) *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci. USA.* 88, 6575-6579; Young, J.W., et al. (1992) *J. Clin. Invest.* 90, 229-237; Koulova, L., et al. (1991) *J. Exp. Med.* 173, 759-762; Reiser, H., et al. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* 144, 4579-4586; LaSalle, J.M., et al., (1991) *J. Immunol.* 147, 774-80; Dustin, M.I., et al., (1989) *J. Exp. Med.* 169, 503; Armitage, R.J., et al. (1992) *Nature* 357, 80-82; Liu, Y., et al. (1992) *J. Exp. Med.* 175, 437-445). One costimulatory pathway involved in T cell activation involves the molecule CD28 on the surface of T cells. This molecule can receive a costimulatory signal delivered by a ligand on B cells or other APCs. Ligands for CD28 include members of the B7 family of B lymphocyte activation antigens, such as B7-1 and/or B7-2 (Freedman, A.S. et al. (1987) *J. Immunol.* 137, 3260-3267; Freeman, G.J. et al. (1989) *J. Immunol.* 143, 2714-2722; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174, 625-631; Freeman, G.J. et al. (1993) *Science* 262, 909-911; Azuma, M. et al. (1993) *Nature* 366, 76-79; Freeman, G.J. et al. (1993) *J. Exp. Med.* 178, 2185-2192). B7-1 and B7-2 are also ligands for another molecule, CTLA4, present on the surface of activated T cells, although the role of CTLA4 in costimulation is unclear.

Delivery of an antigen-specific signal with a costimulatory signal to a T cell leads to T cell activation, which can include both T cell proliferation and cytokine secretion. In contrast, delivery of an antigen-specific signal to a T cell in the absence of a costimulatory signal is thought to induce a state of unresponsiveness or anergy in the T cell, thereby inducing antigen-specific tolerance in the T cell.

Interactions between T cells and B cells play a central role in immune responses. Induction of humoral immunity to thymus-dependent antigens requires "help" provided by T helper (hereafter Th) cells. While some help provided to B lymphocytes is mediated by soluble molecules released by Th cells (for instance lymphokines such as IL-4 and IL-5),

activation of B cells also requires a contact-dependent interaction between B cells and Th cells. Hirohata et al., *J. Immunol.*, 140:3736-3744 (1988); Bartlett et al., *J. Immunol.*, 143:1745-1754 (1989). This indicates that B cell activation involves an obligatory interaction between cell surface molecules on B cells and Th cells. The molecule(s) on the T cell therefore mediates contact-dependent helper effector functions of T cells. A contact-dependent interaction between molecules on B cells and T cells is further supported by the observation that isolated plasma membranes of activated T cells can provide helper functions necessary for B cell activation. Brian, *Proc. Natl. Acad. Sci. USA*, 85:564-568 (1988); Hodgkin et al., *J. Immunol.*, 145:2025-2034 (1990); Noelle et al., *J. Immunol.*, 146:1118-1124 (1991).

A molecule, CD40, has been identified on the surface of immature and mature B lymphocytes which, when crosslinked by antibodies, induces B cell proliferation. Valle et al., *Eur. J. Immunol.*, 19:1463-1467 (1989); Gordon et al., *J. Immunol.*, 140:1425-1430 (1988); Gruber et al., *J. Immunol.*, 142: 4144-4152 (1989). CD40 has been molecularly cloned and characterized. Stamenkovic et al., *EMBO J.*, 8:1403-1410 (1989). A ligand for CD40, gp39 (also called CD40 ligand or CD40L) has also been molecularly cloned and characterized. Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992). The gp39 protein is expressed on activated, but not resting, $CD4^+$ Th cells. Spriggs et al., *J. Exp. Med.*, 176:1543-1550 (1992); Lane et al., *Eur. J. Immunol.*, 22:2573-2578 (1992); Roy et al., *J. Immunol.*, 151:1-14 (1993). Cells transfected with the gp39 gene and expressing the gp39 protein on their surface can trigger B cell proliferation and, together with other stimulatory signals, can induce antibody production. Armitage et al., *Nature*, 357:80-82 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992).

Summary of the Invention

Cell-surface molecules which mediate contact-dependent helper effector functions of T cells are important for inducing immune responses which require T cell help. For example, the interaction of gp39 on T cells with CD40 on B cells plays a central role in activating B cell responses to antigens. The current invention is based, at least in part, on the discovery that cell-surface molecules which mediate contact-dependent helper effector functions also play a critical role in the response of T cells to antigens. In particular, it has been discovered that, under appropriate conditions, interference of an interaction between gp39 on a T cell and a ligand on a cell which is presenting antigen to the T cell can induce antigen-specific T cell tolerance. Accordingly, the cell which presents antigen to the T cell requires an interaction between a gp39 ligand (e.g., CD40) on the cell and gp39 on the T cell to be able to provide signals necessary for activation of the T cell. Inhibition of the interaction between the gp39 ligand and gp39 prevents T cell activation and rather induces antigen-specific T cell tolerance.

The methods of the invention pertain to induction of antigen-specific T cell tolerance. The methods involve contacting a T cell with: 1) a cell which presents antigen to the T cell and has a ligand on the cell surface which interacts with a receptor on the surface of the T cell which mediates contact-dependent helper effector functions; and 2) an antagonist of the receptor on the surface of a T cell which mediates contact-dependent helper effector functions. The antagonist inhibits the interaction of the receptor with it's ligand. A T cell can be contacted with the cell which presents antigen and the antagonist *in vitro*, or alternatively, the cell and the antagonist can be administered to a subject to induce T cell tolerance *in vivo*.

In a preferred embodiment, the receptor on the surface of the T cell which mediates contact-dependent helper effector functions is gp39. In this embodiment, the antagonist is a molecule which inhibits the interaction of gp39 with its ligand on a cell which presents antigen to the T cell. A particularly preferred gp39 antagonist is an anti-gp39 antibody. Alternatively, the gp39 antagonist is a soluble form of a gp39 ligand, for example soluble CD40. The cell which presents antigen to a T cell is preferably a B cell. The B cell can be a small, resting B cell. To induce T cell tolerance to a soluble antigen, the B cell can be contacted with the antigen prior to contact with the T cell (e.g., prior to administration to a subject). In another embodiment, to induce T cell tolerance to alloantigens, the cell which is used to present antigen to the T cell is an allogeneic cell. The allogeneic cell can be, for example, an allogeneic B cell, allogeneic bone marrow, allogeneic spleen cells or allogeneic cells in peripheral blood.

The methods of the current invention can be used, for example, to induce T cell tolerance to a soluble antigen, to induce T cell tolerance a bone marrow transplant or other organ transplant or to inhibit graft-versus-host disease in bone marrow transplantation. In the case of bone marrow transplantation, the transplanted bone marrow cells themselves serve as cells which present antigen to the T cell in the method of the invention. Accordingly, in one embodiment of the invention, acceptance of a bone marrow transplant is promoted by administering to a subject allogeneic bone marrow in conjunction with a gp39 antagonist (e.g., an anti-gp39 antibody).

This invention further pertains to anti-human gp39 monoclonal antibodies capable of inhibiting B cell proliferation, B cell differentiation and T cell responses and to pharmacuetical compositions comprising such antibodies. Anti-human gp39 monoclonal antibodies of the invention are preferred for use in modulating immune responses in general, and particularly for use in inducing antigen-specific T cell tolerance. Preferred antibodies include monoclonal antibodies 3E4, 2H5, 2H8, 4D9-8, 4D9-9, 24-31, 24-43, 89-76 and 89-79, described in Example 6. Particularly preferred antibodies are monoclonal antibodies 89-76 and 24-31. The 89-76 and 24-31 hybridomas, producing the 89-76 and 24-31 antibodies, respectively, were deposited under the provisions of the Budapest Treaty with the American Type Culture Collection, Parklawn Drive, Rockville, Md., on September 2, 1994. The 89-76 hybridoma was assigned ATCC Accession Number ____ and the 24-31 hybridoma was

assigned ATCC Accession Number ____ . The 24-31 and 89-76 antibodies are of the IgG1 isotype.

Accordingly, in one embodiment, the invention provides an anti-human gp39 monoclonal antibody (mAb) of an IgG1 isotype. The anti-human gp39 mAb of the invention can inhibit B cell proliferation in a standard in vitro assay, for example, B cell proliferation induced by treatment of the B cells with interleukin-4 and soluble gp39. Preferably, the anti-human gp39 antibody inhibits B cell proliferation with an IC_{50} (i.e., concentration necessary to inhibit proliferation by 50%) between about 0.01 and 5.0 μg/ml, more preferably between about 0.1 and 2.5 μg/ml, and even more preferably between about 0.1 and 1.25 μg/ml. The anti-human gp39 mAbs of the invention can also inhibit B cell production of IgG, IgM and/or IgA in a standard in vitro assay, for example, Ig production induced by culturing of B cells with activated T cells (e.g., T cells activated by treatment with anti-CD3 antibody). Preferably, the anti-human gp39 antibody inhibits B cell production of IgG, IgM and/or IgA with an IC50 between about 0.01 and 1.0 μg/ml or, more preferably, between about 0.01 and 0.1 μg/ml.

In a preferred embodiment, the anti-human gp39 mAb of the invention binds an epitope recognized by a monoclonal antibody selected from a group consisting of 3E4, 2H5, 2H8, 4D9-8, 4D9-9, 24-31, 24-43, 89-76 and 89-79. More preferably, the anti-human gp39 mAb binds an epitope recognized by monoclonal antibody 24-31 or monoclonal antibody 89-76. The ability of an mAb to bind an epitope recognized by any of the aforementioned antibodies can be determined by standard cross-competition assays. For example, an antibody that binds the same epitope recognized by mAb 24-31 will compete for the binding of labeled 24-31 to activated T cells, whereas an antibody that binds a different epitope than that recognized by mAb 24-31 will not compete for the binding of labeled 24-31 to activated T cells.

The invention also provides pharmaceutical compositions of the anti-human gp39 antibodies of the invention. These compositions typically comprise an anti-human gp39 mAb (e.g., preferably 24-31 or 89-76) and a pharmaceutically acceptable carrier.

Yet another aspect of the invention pertains to nucleic acid encoding an anti-human gp39 mAb (e.g., DNA encoding an immunoglobulin heavy chain or light chain, or portion thereof, of an anti-human gp39 mAb). Such nucleic acid can be isolated from a cell (e.g., hybridoma) producing an anti-human gp39 mAb by standard techniques. For example, nucleic acid encoding the 24-31 or 89-76 mAb can be isolated from the 24-31 or 89-76 hybridoma, respectively, by cDNA library screening, PCR amplification or other standard technique. Nucleic acid encoding an anti-human gp39 mAb chain can be manipulated by standard recombinant DNA techniques to produce recombinant anti-human gp39 mAbs, for example, chimeric or humanized anti-human gp39 mAbs.

Moreover, nucleic acid encoding an anti-human gp39 mAb can be incorporated into an expression vector and introduced into a host cell to facilitate expression and production of recombinant forms of anti-human gp39 antibodies.

Brief Description of the Drawings

Figure 1 is a graphic representation of T cell tolerance to a protein antigen induced by *in vivo* anti-gp39 treatment. T cells responses were measured *in vitro* upon challenge with an antigen which previously was administered *in vivo* on antigen-pulsed B cells either with or without an anti-gp39 antibody.

Figure 2 is a graphic representation of T cell tolerance to allogeneic B cells induced by *in vivo* anti-gp39 treatment. T cells responses were measured *in vitro* upon challenge with allogeneic B cells which previously were administered *in vivo* either with or without an anti-gp39 antibody.

Figure 3A is a graphic representation of the inhibition of primary allogeneic CTL responses induced by allogeneic B cells when recipient animals are treated with anti-gp39 antibody. Groups represented are untreated mice (■), anti-gp39 treated mice (Δ) and spleen cells from unprimed Balb/c mice (●; used as negative control effector cells).

Figures 3B and 3C are graphic representations of the inhibition of primary allogeneic CTL responses induced by LPS-treated B cell blasts when recipient animals are treated with anti-gp39 antibody. Panels B and C represent two independent experiments. Groups represented are LPS blasts *in vivo* without treatment (▶), LPS blasts *in vivo* with anti-gp39 treatment (●), resting B cells *in vivo* without treatment (○) and resting B cells *in vivo* with anti-gp39 treatment (□).

Figure 4A is a graphic representation of the inhibition of secondary allogeneic CTL responses induced by allogeneic B cells when recipient animals are treated with anti-gp39 antibody. Effector groups shown are: HIg treated recipients (●), naive Balb/c (▶) and anti-gp39 treated recipients (■). Corresponding syngeneic response (Balb/c cells stimulated with Balb/c cells) are indicated by open symbols.

Figure 4B is a graphic representation of the specificity of inhibition of allogeneic CTL responses by anti-gp39 treatment. Shown are CTL responses against $H\text{-}2^k$ targets by naive Balb/c cells (○) or by cells tolerized to $H\text{-}2^b$ by administration of $H\text{-}2^b$ haplotype B cells and anti-gp39 (■).

Figure 5 is a bar graph depicting the number of splenocytes in a host which has received a bone marrow transplant at various times after transfer of the bone marrow to the host either with or without treatment with an anti-gp39 antibody.

Figure 6A is a graphic representation of the concentration of IgA produced by splenic B cells *in vitro* after removal from mice which received a bone marrow transplant either with or without *in vivo* anti-gp39 treatment. Splenic B cells were removed and antibody production measured either 7 or 14 days after bone marrow transplantation.

Figure 6B is a graphic representation of the concentration of IgG1 produced by splenic B cells *in vitro* after removal from mice which received a bone marrow transplant either with or without *in vivo* anti-gp39 treatment. Splenic B cells were removed and antibody production measured either 7 or 14 days after bone marrow transplantation.

Figure 7A is a graphic representation of the serum IgE concentrations in mice which received a bone marrow transplant either with or without *in vivo* anti-gp39 treatment at various times after bone marrow transfer.

Figure 7B is a graphic representation of the serum anti-DNA antibody concentrations in mice which received a bone marrow transplant either with or without *in vivo* anti-gp39 treatment at various times after bone marrow transfer.

Figures 8A and 8B are graphic representations depicting the cytolytic activity *in vitro* of cytotoxic T cells from mice which received a bone marrow transplant either with or without *in vivo* anti-gp39 treatment at different effector to target cell ratios (E:T ratio). Panels A and B represent two independent experiments.

Figures 9A, 9B and 9C are flow cytometic profiles depicting the staining of 6 hour activated human peripheral blood lymphocytes with either CD40Ig (panel A), mAb 4D9-8 (panel B) or mAb 4D9-9 (panel C).

Figures10A, 10B and 10C are flow cytometic profiles depicting the staining of 6 hour activated human peripheral blood lymphocytes cultured in the presence of cycloporin A stained with either mAb 4D9-8 (panel A), mAb 4D9-9 (panel B) or CD40Ig (panel C).

Figures 11A and 11B are flow cytometric profiles depicting the staining of 6 hour activated human peripheral blood lymphocytes with CD40Ig in the presence of unlabeled mAb 4D9-8 (panel A) or unlabeled mAb 4D9-9 (panel B).

Figure 12 is a graphic representation of the inhibition of human B cell proliferation induced by soluble gp39 and IL-4 when cells are cultured in the presence of anti-human gp39 mAbs 4D9-8, 4D9-9, 24-31, 24-43, 89-76 or 89-79.

Figure 13 is a graphic representation of the inhibition of an allo-specific mixed lymphocyte response when cells are cultured in the presence of anti-human gp39 mAbs 24-31 or 89-79.

Detailed Description of the Invention

This invention features methods for inducing antigen-specific T cell tolerance. The methods involve contacting a T cell with 1) a cell which presents antigen to the T cell and has a ligand on the cell surface that interacts with a receptor on the surface of the T cell which mediates contact dependent helper effector functions, and 2) an antagonist of the receptor on the T cell which inhibits interaction of the receptor an the ligand. As defined herein, a molecule or receptor which mediates contact dependent helper effector functions is one which is expressed on a Th cell and interacts with a ligand on an effector cell (e.g., a B cell), wherein the interaction of the receptor with its ligand is necessary for generation of an

effector cell response (e.g., B cell activation). In addition to being involved in effector cell responses, it has now been found that such a molecule is involved in the response of the T cell to antigen.

A preferred molecule on a T cell which mediates contact-dependent helper effector function is gp39. Accordingly, in preferred embodiments, the methods of the invention involve contacting a T cell with a cell which presents antigen and a gp39 antagonist. Accordingly, the cell used to present antigen is one which interacts with gp39 on the surface of a T cell to activate the T cell (i.e. deliver the necessary signals for T cell activation to the T cell). For example, the cell can be a B cell which expresses CD40 and presents antigen to the T cell. By inhibiting an interaction between a gp39 ligand on the cell presenting antigen with gp39 on the T cell, the T cell is not activated by the presented antigen but rather becomes tolerized to the antigen.

The methods of the invention can be used to induce T cell tolerance to an antigen *in vivo*. For example, a cell which presents antigen to a T cell can be administered to a subject in conjunction with an antagonist of a receptor expressed on the T cell which mediates contact dependent helper effector function (e.g. a gp39 antagonist). The methods of the invention can further be used to tolerize a T cell to an antigen *in vitro* by contacting the T cell *in vitro* with a cell which presents antigen to the T cell together with an antagonist of a receptor expressed on the T cell which mediates contact dependent helper effector function (e.g. a gp39 antagonist). T cells tolerized *in vitro* can then be administered to a subject. The methods of the invention can be used to tolerize T cells in a subject to a specific antigen, or to transplanted cells, such as allogeneic bone marrow (e.g., in bone marrow transplantation). The methods of the invention are also useful for inhibiting graft versus host disease in bone marrow transplantation.

Various aspects of the invention are described in further detail in the following subsections.

I. gp39 Antagonists

According to the methods of the invention, a gp39 antagonist is contacted with a T cell (e.g. administered to a subject) to interfere with the interaction of gp39 on a T cell with a gp39 ligand on an antigen presenting cell, such as a B cell. A gp39 antagonist is defined as a molecule which interferes with this interaction. The gp39 antagonist can be an antibody directed against gp39 (e.g., a monoclonal antibody against gp39), a fragment or derivative of an antibody directed against gp39 (e.g., Fab or F(ab)'2 fragments, chimeric antibodies or humanized antibodies), soluble forms of a gp39 ligand (e.g., soluble CD40), soluble forms of a fusion protein of a gp39 ligand (e.g., soluble CD40Ig), or pharmaceutical agents which disrupt or interfere with the gp39-CD40 interaction.

A. Antibodies

A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of gp39 protein or protein fragment (e.g., peptide fragment) which elicits an antibody response in the mammal. A cell which expresses gp39 on its surface can also be used as the immunogen. Alternative immunogens include purified gp39 protein or protein fragments. gp39 can be purified from a gp39-expressing cell by standard purification techniques; gp39 cDNA (Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992)) can be expressed in a host cell, e.g., bacteria or a mammalian cell line, and gp39 protein purified from the cell culture by standard techniques. gp39 peptides can be synthesized based upon the amino acid sequence of gp39 (disclosed in Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992)) using known techniques (e.g. F-moc or T-boc chemical synthesis). Techniques for conferring immunogenicity on a protein include conjugation to carriers or other techniques well known in the art. For example, the protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (*Nature* (1975) 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., *Immunol. Today* (1983) 4:72), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. *Monoclonal Antibodies in Cancer Therapy* (1985) (Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., *Science* (1989) 246:1275). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the protein or peptide and monoclonal antibodies isolated.

The term antibody as used herein is intended to include fragments thereof which are specifically reactive with a gp39 protein or peptide thereof or gp39 fusion protein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having an anti-gp39 portion.

When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes gp39. See, for example, Morrison et al., *Proc. Natl. Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. It is expected that such chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

For human therapeutic purposes the monoclonal or chimeric antibodies specifically reactive with a gp39 protein or peptide can be further humanized by producing human variable region chimeras, in which parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today*, 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

Another method of generating specific antibodies, or antibody fragments, reactive against a gp39 protein or peptide is to screen expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with a gp39 protein or peptide. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries. See for example Ward et al., *Nature*, 341: 544-546: (1989); Huse et al., *Science*, 246: 1275-1281 (1989); and McCafferty et al., *Nature*, 348: 552-554 (1990). Screening such libraries with, for example, a gp39 peptide can identify imunoglobin fragments reactive with gp39. Alternatively, the SCID-hu mouse (available from Genpharm) can be used to produce antibodies, or fragments thereof.

Methodologies for producing monoclonal antibodies directed against gp39, including human gp39 and mouse gp39, and suitable monoclonal antibodies for use in the methods of the invention, are described in further detail in Example 6. Particularly preferred anti-human gp39 antibodies of the invention are mAbs 24-31 and 89-76, produced respectively by

hybridomas 24-31 and 89-76. The 89-76 and 24-31 hybridomas, producing the 89-76 and 24-31 antibodies, respectively, were deposited under the provisions of the Budapest Treaty with the American Type Culture Collection, Parklawn Drive, Rockville, Md., on September 2, 1994. The 89-76 hybridoma was assigned ATCC Accession Number ____ and the 24-31 hybridoma was assigned ATCC Accession Number ____ .

Recombinant anti-gp39 antibodies, such as chimeric and humanized antibodies, can be produced by manipulating nucleic acid (e.g., DNA) encoding an anti-gp39 antibody according to standard recombinant DNA techniques. Accordingly, another aspect of this invention pertains to isolated nucleic acid molecules encoding immunoglobulin heavy or light chains, or portions thereof, reactive with gp39, particularly human gp39. The immunoglobulin-encoding nucleic acid can encode an immunoglobulin light or heavy chain variable region, with or without a linked heavy or light chain constant region (or portion thereof). Such nucleic acid can be isolated from a cell (e.g., hybridoma) producing an anti-human gp39 mAb by standard techniques. For example, nucleic acid encoding the 24-31 or 89-76 mAb can be isolated from the 24-31 or 89-76 hybridoma, respectively, by cDNA library screening, PCR amplification or other standard technique. Following isolation of, and possible further manipulation of, Moreover, nucleic acid encoding an anti-human gp39 mAb can be incorporated into an expression vector and introduced into a host cell to facilitate expression and production of recombinant forms of anti-human gp39 antibodies.

B. Soluble Ligands for gp39

Other gp39 antagonists which can be used to induce T cell tolerance are soluble forms of a gp39 ligand. A monovalent soluble ligand of gp39, such as soluble CD40 can bind gp39, thereby inhibiting the interaction of gp39 with CD40 on B cells. The term "soluble" indicates that the ligand is not permanently associated with a cell membrane. A soluble gp39 ligand can be prepared by chemical synthesis, or, preferably by recombinant DNA techniques, for example by expressing only the extracellular domain (absent the transmembrane and cytoplasmic domains) of the ligand. A preferred soluble gp39 ligand is soluble CD40. Alternatively, a soluble gp39 ligand can be in the form of a fusion protein. Such a fusion protein comprises at least a portion of the gp39 ligand attached to a second molecule. For example, CD40 can be expressed as a fusion protein with immunoglobulin (i.e., a CD40Ig fusion protein). In one embodiment, a fusion protein is produced comprising amino acid residues of an extracellular domain portion of the CD40 molecule joined to amino acid residues of a sequence corresponding to the hinge, CH2 and CH3 regions of an immunoglobulin heavy chain, e.g., Cγ1, to form a CD40Ig fusion protein (see e.g., Linsley et al. (1991) *J. Exp. Med.* 1783:721-730; Capon et al. (1989) *Nature* 337, 525-531; and Capon U.S. 5,116,964). The fusion protein can be produced by chemical synthesis, or, preferably by recombinant DNA techniques based on the cDNA of CD40 (Stamenkovic et al., *EMBO J.*, 8:1403-1410 (1989)).

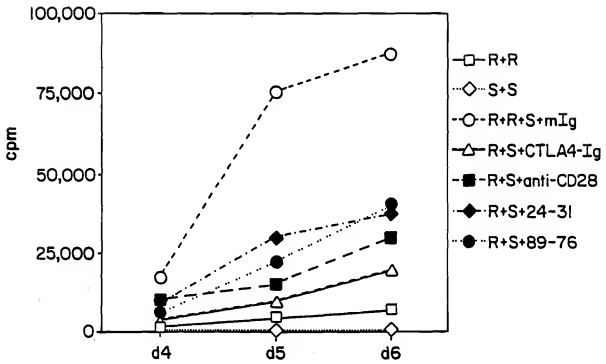
II. Cells for Induction of Antigen-Specific Tolerance

The current invention is based, at least in part, on the discovery that presentation of an antigen to a T cell by a cell which both presents antigen and interacts with gp39 results in antigen-specific T cell tolerance when the antigen is presented to the T cell in the presence of a gp39 antagonist. Cells which are capable of inducing T cell tolerance by this mechanism include those which present antigen to a T cell and require an interaction between a gp39 ligand on the cell and gp39 on the T cell to deliver the necessary signals for T cell activation to the T cell. Inhibition of this interaction prevents T cell activation by the presented antigen and, rather, induces antigen-specific tolerance in the T cell. Interference with activation of the T cell via gp39 may prevent the induction of costimulatory molecules on the antigen presenting cell (e.g., B7 family molecules on an antigen presenting cell such as a B cell) so that the antigen presenting cell delivers only an antigenic signal in the absence of a costimulatory signal, thus inducing tolerance.

Accordingly, in the methods of the invention, a cell which presents antigen is administered to a recipient subject. The phrase "cell which presents antigen" and "antigen presenting cell" are used interchangeably herein and are intended to encompass cells which can present antigen to T cells of the recipient and includes B lymphocytes, "professional" antigen presenting cells (e.g., monocytes, dendritic cells, Langerhan cells) and other cells which present antigen to immune cells (e.g., keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes). Furthermore, it is preferable that the antigen presenting cell have a reduced capacity to stimulate a costimulatory signal in recipient T cells. For example, the antigen presenting cell may lack expression of or express only low levels of costimulatory molecules such as the B7 family of proteins (e.g., B7-1 and B7-2). Expression of costimulatory molecules on potential antigen presenting cells to be used in the method of the invention can be assessed by standard techniques, for example by flow cytometry using antibodies directed against costimulatory molecules.

Preferred antigen presenting cells for inducing T cell tolerance are lymphoid cells, for example peripheral blood lymphocytes or splenic cells. Preferred lymphoid cells for inducing T cell tolerance are B cells. B cells can be purified from a mixed population of cells (e.g., other cell types in peripheral blood or spleen) by standard cell separation techniques. For example, adherent cells can be removed by culturing spleen cells on plastic dishes and recovering the non-adherent cell population. T cells can be removed from a mixed population of cells by treatment with an anti-T cell antibody (e.g., anti-Thy1.1 and/or anti-Thy1.2) and complement. In one embodiment, resting lymphoid cells, preferably resting B cells, are used as the antigen presenting cells. Resting lymphoid cells, such as resting B cells, can be isolated by techniques known in the art, for example based upon their small size and density. Resting lymphoid cells can be isolated for example by counterflow centrifugal elutriation as described in Tony, H-P. and Parker, D.C. (1985) *J. Exp. Med.* 161:223-241.

FIG. 13


100,000
75,000
50,000
25,000
0
cpm
d4
d5
d6
R+R
S+S
R+R+S+mIg
R+S+CTLA4-Ig
R+S+anti-CD28
R+S+24-31
R+S+89-76

TERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/09953

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/17 A61K35/14 A61K35/28
//(A61K39/395,35:14),(A61K39/395,35:28),(A61K38/17,35:14)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 555 880 (BRISTOL-MYERS SQUIBB COMPANY ET AL.) 18 August 1993 see column 14, line 4 - column 20, line 41 see claims ---	1-58
A	THE JOURNAL OF IMMUNOLOGY, vol.151, no.4, 15 August 1993, BALTIMORE MD, USA pages 1777 - 1788 B. CASTLE ET AL. 'Regulation of expression of the ligand for CD40 on T helper lymphocytes.' see abstract see page 1780, right column --- -/--	1-58

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 1994	28.12.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNA. ONAL SEARCH REPORT

International application No.
PCT/US 94/09953

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although all claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CLINICAL IMMUNOLOGY, vol.13, no.3, May 1993, NEW YORK NY, USA pages 165 - 174 L. MARSHALL ET AL. 'The molecular basis for T cell help in humoral immunity: CD40 and its ligand, gp39.' see page 165, right column, line 8 - line 29; figure 1 see page 167, right column, line 17 - line 28 see page 168, right column, line 49 - page 169, left column, line 38 ---	1-58
A	IMMUNOLOGY TODAY, vol.13, no.11, November 1992, AMSTERDAM, THE NETHERLANDS pages 431 - 433 R. NOELLE ET AL. 'CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation.' see the whole document ---	1-58
P,A	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol.178, no.5, 1 November 1993, NEW YORK NY, USA pages 1567 - 1575 T. FOY ET AL. 'In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39.' see abstract see page 1572, left column, line 8 - line 42 -----	1-58

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

International Application No
PCT/US 94/09953

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0555880	18-08-93	AU-A-	3298893	19-08-93
		CA-A-	2089229	15-08-93
		JP-A-	6220096	09-08-94